

OHIO STATE UNIVERSITY

# Effect of Elastin-Like Polypeptide Tag Length on the Purification of Antimicrobial Peptides

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An Honors Thesis Presented in Accordance with the Requirements for Graduation  
with Distinction in Chemical and Biomolecular Engineering

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## ABSTRACT

Due to the observed increase in antibiotic resistance of bacteria, there has been a search for new types of therapeutics to combat this phenomenon, which is presenting one of the greatest challenges to Global Health Programs. Antimicrobial peptides, small proteins with less than 100 amino acid residues, are one promising field of new candidate molecules for antimicrobials. One obstacle to their study is a reliable method of recombinant purification in *E. coli*. Higher yields of the peptides are necessary for their efficient large scale study. A fusion protein consisting of a self-cleaving intein and a precipitation tag known as Elastin-Like Peptide (ELP) is one possible purification scheme for increasing the yield of active antimicrobial peptide. Previous work in the lab of Dr. David Wood created a series of pET vectors containing ELP tags of reducing size length from 550 to 50 amino acids. Using a reduced tag size, it should theoretically be possible to increase the yield of peptide due to increased metabolic efficiency. In this study two peptides, Equine Defensin  $\alpha$ -1 and Ctriporin, were introduced into the pET-E-I vector and expressed *E. coli* BLR. The protein was purified using the Inverse Transition Cycling (ITC) method and assessed for purity on Tricene-SDS 10-25% gradient precast gels. The activity of the peptides against *S. equi* and *E. coli* was tested using broth microdilution and radial diffusion tests. The peptides, when correctly purified, were not active against the bacteria in both tests. One possible reason for this inactivation is the use of ammonium sulfate as the salt in the ITC method instead of sodium chloride. In the future the peptides should be purified and tested while using sodium chloride as the precipitating salt for the ITC method.

## Acknowledgements

I would like to thank Dr. David Wood for allowing me to conduct research in his lab and helping me with every step of this process. I would also like to thank the other members of the Wood Group for their help during my time as part of their group. I would especially like to thank Dr. Richard Lease and Michael Coolbaugh for the expertise and knowledge they shared with me.

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## 1. Introduction

In the last two decades the emergence of multi-drug resistant pathogenic bacteria has become a major concern for the medical field. “Contributing factors to this development include the inappropriate use of antibiotics, such as the use of powerful, broad spectrum antibiotics, the presence of antibiotics in the food/livestock industry, and from the inclusion of antibiotics in household products.”<sup>1</sup> With the appearance of highly resistant MRSA and extensively-drug resistant tuberculosis, it is critical to identify new candidates for antibacterial drugs. Antimicrobial peptides (AMPs) may be a potential solution to this problem. Of their many benefits, some antimicrobial peptides offer a broad spectrum of activity, while other peptides have highly specific activities for specific types of pathogens. They are also present in almost every life-form, meaning that there is a huge pool of potential molecules. Finally, it has been demonstrated that the speed and ability of bacteria to acquire resistance to antimicrobial peptides is reduced when compared to conventional antibiotics, which is a huge advantage of antimicrobial peptides over current drugs.<sup>2</sup>

However, there are some roadblocks to their use as common antibiotics. One of the major setbacks is that there is a need for a simple and reliable method for high-yield production of AMPs at a laboratory and clinical scale. Chemical synthesis is currently too expensive to be an option for large scale production, and purifying AMPs from their natural sources is even more complicated and expensive. The best currently available option is the recombinant expression of AMPs in a bacterium such as *E. coli*. Recombinant purification of these peptides in *E. coli* is not without its own challenges. These peptides have the potential to be fatal to the producing cell and can be subject to high proteolytic activity. In addition, the peptides are very unstable and can easily be inactivated by temperature fluctuations and other typical phenomena in purification. Expressing the peptides as part of fusion protein with a removable purification tag is one possible method for the production of AMPs in *E. coli*.

Several types of purification methods are available for recombinant production of AMPs. One commonly used method is the expression of the peptide as a fusion protein with an affinity tag protein. Affinity tags are some of the major workhorses of the pharmaceutical industry.<sup>3</sup> Affinity resin proteins typically used for *E. coli* production systems include the chitin binding protein (CBD), the maltose binding protein (MBP), and the peptide tag hexahistidine (His). The main advantage of affinity resin tags is that they can provide hundred or thousand-fold purification steps without the need to remove cellular components such as DNA and other host cell proteins (HCPs) from the clarified lysate using a very generalized protocol.<sup>4</sup> Affinity tags also have disadvantages, especially in the area of cost. Table 1 below is a comparison of several common affinity resin binding proteins. For all of the references shown, the binding capacities are less than 100 mg/ml. In addition, to purify 100 mg of protein, the resin cost will be over \$100 for that purification. The resins can be regenerated for repeated use, driving down the overall cost of the protein, but will become fouled by cell debris over time. In addition to fouling, the resin itself chemically degrades with repeated use. Finally, the tag itself may interfere with the proper functioning of the target protein. This requires that affinity tag must be removed, requiring the use of costly proteases.<sup>5</sup> Antimicrobial peptides have been purified using affinity resins with success.<sup>3, 6</sup> However, yields of the peptide are low and the elimination of the need for a column would be an advantage for other purification methods.

**Table 1:** Comparison of affinity tags for the purification of proteins in *E. coli*.<sup>4</sup>

Tag	Size (aa)	Resin	Eluting agent	Source	Capacity	Cost	Cost/10 mg
MBP	396	Amylose	Maltose	Biolabs	3 mg/ml	\$105/10 ml	\$12
HIS	6	Talon	Imidazole	Clontech	5–14 mg/ml	\$220/25 ml	\$18
		Ni-NTA	Imidazole	Qiagen	5–10 mg/ml	\$257/25 ml	\$21
GST	218	GSH-Sepharose	Glutathione	Amersham	10 mg/ml	\$396/25 ml	\$36
CBP	28	Calmodulin affinity	EGTA	Stratagene	2 mg/ml	\$227/10 ml	\$114
STR (Strep II)	8	Strep-Tactin-Sepharose	Desthiobiotin	IBA	50–100 nmol/ml	\$1100/25 ml	\$293
FLAG	8	Anti-FLAG M2 MAb agarose	FLAG peptide	Sigma	0.6 mg/ml	\$1568/25 ml	\$1045
HPC	12	Anti-Protein C MAb matrix	EDTA	Roche	2–10 nmol/ml	\$299/1 ml	\$4983
CYD	5	InaD	DTT	N/A	>0.2 mg/ml	N/A	N/A

The cost of tag removal can be decreased by the use of a protein splicing element known as an intein. Inteins “have the unique ability to excise themselves from within a translated host protein.”<sup>7</sup> The

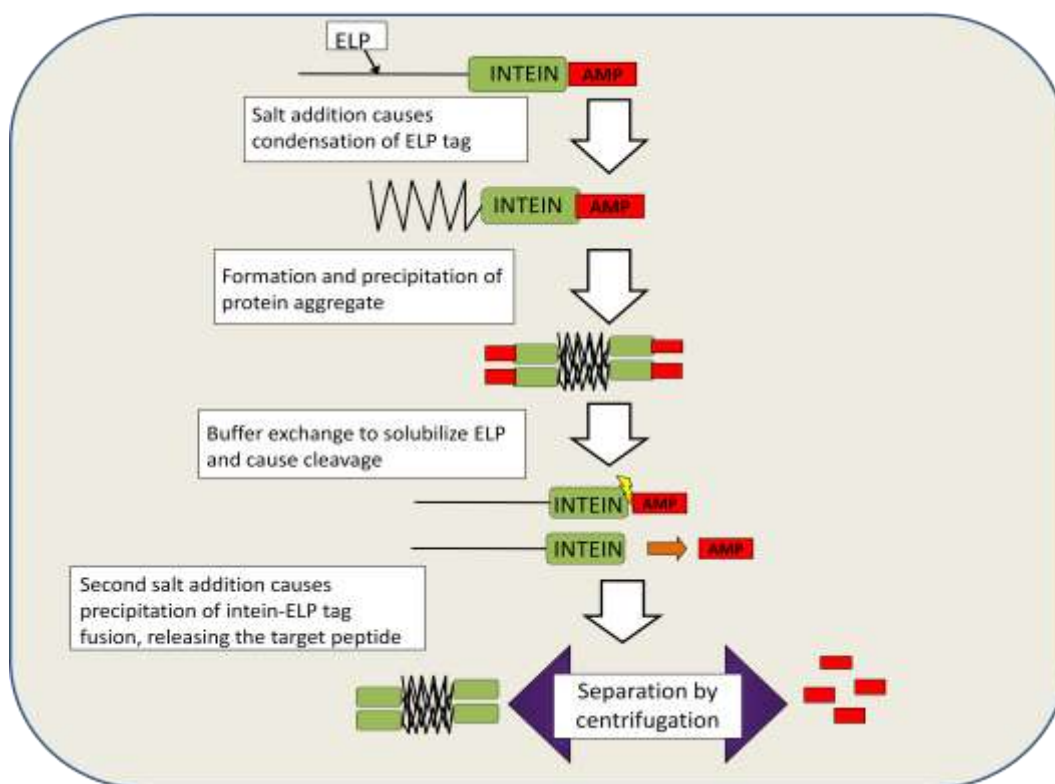
intein is expressed as part of the precursor protein and then cleaves itself from the expressed protein, resulting in two new proteins, one of which contains the intein sequence of amino acids.<sup>8</sup> Inteins have been modified for use in protein purification, creating variants that are N- or C-terminal cleaving.<sup>3</sup> These proteins can be expressed with a purification tag to yield a method for simple bioseparations.<sup>9</sup>

AMPs have been purified using peptide-intein-affinity tag fusion proteins. Chen, et al. purified ABP-CM4 and ABP-CMt *in E. coli* using the Sce VMA intein system which contains the chitin-binding domain protein. The fusion method achieved yields of ABP-CM4 of 2.1 mg of protein from 500 ml of bacterial culture at 95% purity and proved that the peptides did have antimicrobial activity. In other research, active His-tagged cathelicidin derivatives were purified but at similarly low yields (<1 mg/L).<sup>10</sup> If an intein system is to be used, yield must be increased.

The elimination of the affinity column step to the purification could improve the yield by removing a source of product loss in the purification steps. The elastin-like peptide, a thermally responsive fusion tag, is a method of protein purification that does not require column chromatography.<sup>11</sup> ELP-intein fusions have been developed that successfully purified green fluorescent protein and  $\beta$ -galactosidase.<sup>12</sup> Several antimicrobial peptides have been recombinantly expressed using the ELP-intein tag including human beta-defensin-4 and moricin CM4.<sup>13</sup> The yields of these two proteins were deemed unsatisfactory by the authors however.

In this research, the ELP-intein system developed by Dr. David W. Wood will be used to purify two antimicrobial peptides. Purification of the ELP intein-peptide fusion can be achieved through Inverse Transition Cycling (ITC), and tag removal and purification of the final peptide can be achieved by the addition of a cleaving buffer to the solution containing the expressed fusion protein, followed by another round of salt addition.<sup>14</sup> Figure 1 below outlines the basic method of purification of an AMP using the ELP-intein purification tag.





**Figure 1:** The Inverse Transition Cycling method used to purify a target protein developed and used by the Wood group.

In related work, it has been demonstrated that shortening the length of the ELP tag can improve the yield of a fusion protein.<sup>15</sup> Shortening the tag length increases the overall metabolic efficiency in the bacterial cell. The original ELP tag contains 11 repeats of the amino acid sequence (VPGXG)<sub>10</sub>, equal to 550 amino acids. The intein itself is C-terminal and approximately 165 AA in length. With a 40 AA long peptide, decreasing the tag length of ELP to 200 AA represents a reduction of over 40% of the number of AA necessary to produce the protein. The cell will therefore take a shorter time to produce each fusion protein and use fewer resources, theoretically allowing higher yields of the AMP target protein.

The two AMPs that will be used in this experiment were selected based on size and availability. The DNA sequence for AMP Defensin  $\alpha$ -1 (DEFA1), an AMP found in the digestive tract of horses, was provided as part of a collaborative effort with a group in France. It is a positively charged peptide with a length of 46 AA in its mature, active form. The second peptide chosen was Ctriporin (Ctr), a small peptide found in the venom of an Asiatic scorpion. It has antimicrobial properties against Methicillin-

Resistant *Staphylococcus aureus* (MRSA), one of the selection factors, and was selected for its small size in comparison to DEFA1, having a length of 19 AA.<sup>16</sup> Table 2 below gives the basic specification for each peptide, including activity against *S. aureus* and *E. coli*.

**Table 2:** Table of the properties of the two peptides in this research

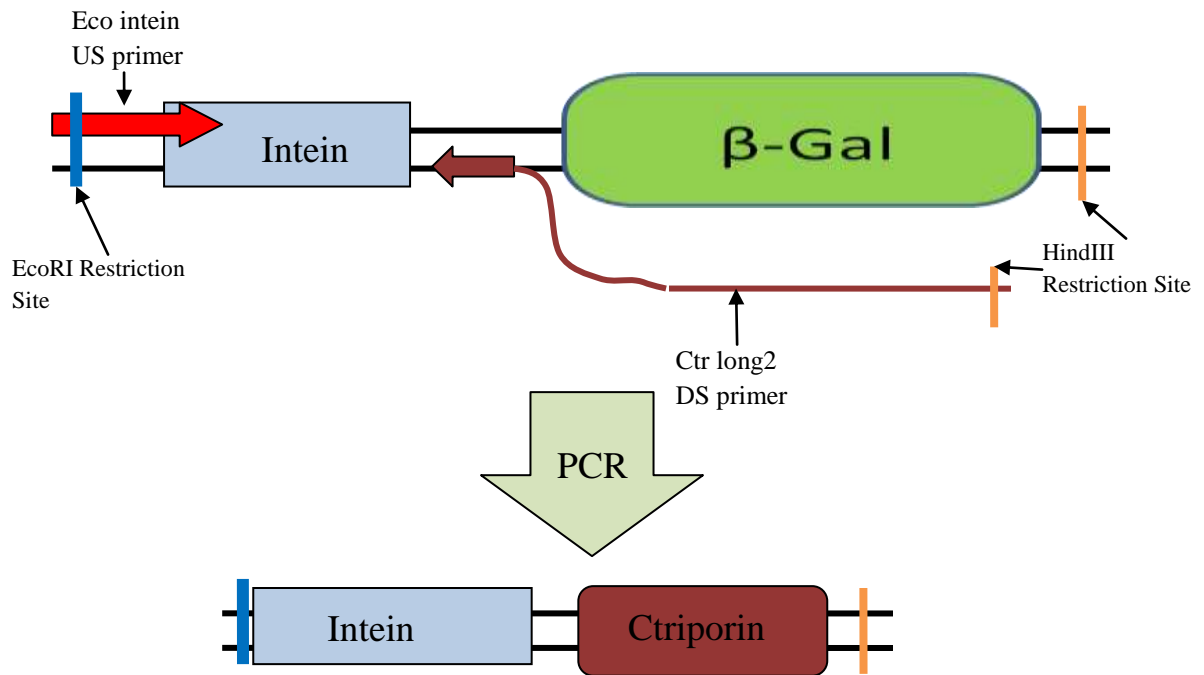
Name	Abbreviation	Mature Peptide Sequence	MIC for <i>S. aureus</i>	MIC for <i>E. coli</i>
Defensin alpha-1	DEFA1	AREASKSLIGTASCTCRRWICRW GERHSGKCIDQKGSTYRLCCRR	6.1 $\mu$ M	0.4 $\mu$ M
Ctriporin	Ctr	FLWGLIPGAISAVTSLIKK	5 ug/ml	No activity

## 2. Materials and Methods

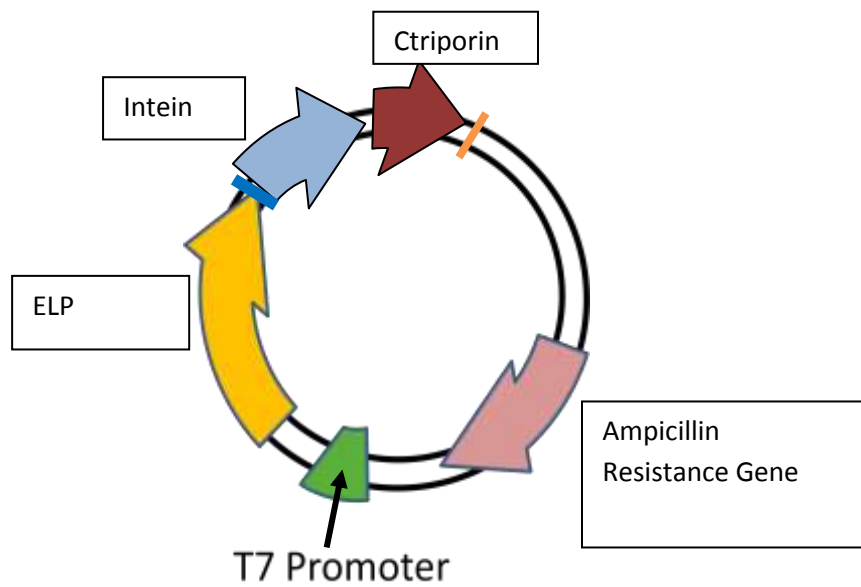
### 2.1. Construction of ELP-Intein Vectors

Previous work by Robert Wensing in Dr. Woods lab created pET vectors with the DNA sequence for the ELP-Intein fusion containing DEFA1 at several different lengths, hereafter referred to as E<sub>x</sub>I-(target protein), where *x* is the number of repeats of the 50 AA sequence compromising ELP. The E<sub>x</sub>I-DEFA1 constructs created by Robert Wensing were in lengths of 1, 2, 4, 5, 6, 8, 9, 10 and 11 repeats of ELP. For this research, E<sub>11</sub>I-DEFA1 and E<sub>4</sub>I-DEFA1 were used.

The vector for EI-Ctr was created through PCR by using a long primer encoding the full DNA sequence of Ctr and an primer with a sequence beginning at the EcoRI site ahead of the intein on a vector containing the E<sub>11</sub>I-β-Galactosidase gene. In addition to the entire sequence of Ctr, the 100 bp Ctr long2 DS primer contained the sequence of the last 24 BP of the self cleaving intein and a modified 3' end with a HindIII restriction site. This long primer was designed to replace the β-gal gene during a PCR reaction as part of the fragment attached to the intein. Figure 2 below is a graphic of the PCR scheme used to design the Intein-Ctr fragment.



**Figure 2:** PCR design of Intein-Ctriporin Fragment.



**Figure 3:** pET EI-Ctr Vector

The two primers, Ctr long2 DS and Eco intein US were combined in a 50  $\mu$ L PCR reaction with pET E<sub>11</sub>I- $\beta$ gal in a molar excess of  $\sim$ 100:1 primer:vector. The conditions for the reaction were a 98°C melting step for 15s, 65°C annealing step for 15s, and a 72°C extension step for 10s. The resulting 600 bp

fragment was digested with EcoRI and HindIII and the cut fragment was excised from a 1% m/v agarose (SeaKem) gel using a QIAquick Gel Extraction kit from Qiagen. The pET E<sub>11</sub>I-βgal was also cut and purified using the same enzymes and gel to serve as the vector for DNA transfection to the cells. The purified 600 bp and 7000 bp fragments with “sticky ends” were ligated using T4 DNA Ligase (New England Biolabs) in a 10:1 ratio of insert to backbone for 24hrs at 16°C. Figure 3 shows the gene structure of the final pET E<sub>11</sub>I-Ctr vector.

The product of the ligation reaction was transformed into Z-Competent *E. coli* DH5α cells according to the Z-Competent *E. coli* Cell Protocol (Zymo Research). Following this transformation, the cells were plated onto Luria Bertani (LB) agar containing 50 µg/ 100 ml of ampicillin to select for the transformed bacteria. Isolated colonies from the transformation were inoculated into 4 mL of LB broth and shaken at 200 RPM overnight at 37°C. The plasmids the clones contained were harvested with the QIAprep Spin Miniprep Kit (Qiagen). The clones were verified by digest map checks using restriction enzymes pairs EcoRI and HindIII, and AgeI and HindIII (New England Biolabs). The confirmed pET E<sub>11</sub>I-Ctr vector recovered in the miniprep was used to transform Z-Competent *E. coli* BLR strains for high-expression production of protein.

## **2.2 Expression of protein**

Once the correct strains of *E. coli* BLR pET E<sub>11</sub>I-DEFA1 and pET E<sub>11</sub>I-Ctr had been created, they were stored at -80°C. Cells were plated fresh from the freezer onto LB media containing 50 µg/100 mL of ampicillin and incubated at 37°C for 20 hours. An isolated colony was selected and used to inoculate 4 mL of LB media, which was then shaken overnight at 200 RPM and 37°C. A 1 mL sample of the bacterial suspension was used to inoculate 100 mL of Terrific Broth (TB) media. The culture was shaken for 4 hrs at 37°C, cooled to 16°C, and then induced with 500 µL of 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The induced culture was shaken for 20 hrs at 16°C.

At the end of expression, the culture was centrifuged for 10 min at 10,000g and 4°C, resuspended in lysis buffer (10 mM Tris-HCl, 2 mM EDTA, pH 8.5) and frozen overnight at -4°C. The pellet was thawed and the cells inside were lysed by sonication for 3 rounds x 15s at 5W RMS and the sample was centrifuged again at 14,000 g and 4°C. The supernatant was withdrawn and the pellet discarded. A sample of the supernatant (Clarified Lysate) was taken at this point to be examined on a Tricene-SDS 10-25% Gradient gel (NuSep). In accordance with a revised ELP purification protocol based on salt optimization an equal amount of .8M ammonium sulfate ((NH<sub>4</sub>)<sub>2</sub>H<sub>2</sub>SO<sub>4</sub>) was added to the supernatant and allowed to warm to room temperature (RT) to induce precipitation of the ELP tag for 10 minutes.<sup>17</sup> The solution was centrifuged again for 5 minutes at 14,000 g at RT and a sample was taken, called the Soluble Contaminants (SC).

The pellet was washed in 0.4M ammonium sulfate and resuspended at in 4°C cleaving buffer (PBS buffer + 40 mM Bis-Tris, 2 mM EDTA, pH 6.2) and centrifuged at 14,000g to remove any insoluble impurities. A sample was taken, the purified precursor (PP). The sample was then allowed to cleave overnight at RT. The next morning a sample of the cleaved product, the post cleavage (PC) sample, was taken. An equal amount of 0.8 M ammonium sulfate was added to the sample, which was then allowed to sit at RT for 10 min. The sample was then centrifuged at 14,000 g and the supernatant contained the purified protein product. A sample of this final product (FP) was taken for Tricene-SDS gel analysis. The final product was then frozen at -4°C for future use. Protein concentrations were determined using an A<sub>280</sub> reading. Samples were run on a Tricene-SDS-PAGE for characterization and to assess purity.

The ammonium sulfate purification method was also compared to the original protocol where NaCl was used to precipitate the tag. The results were compared side-by-side on a Tricene-SDS-PAGE.

## **2.3 Activity Assays**

### **2.3.1 Broth Microdilution Assay**

To test the activity of the purified proteins, two activity tests were conducted. For DEFA1, a broth microdilution assay was conducted. For DEFA1 and Ctriporin, a radial diffusion assay was conducted.

The broth microdilution assay was performed in the manner described by Otvos and Cudic.<sup>18</sup> To prepare for the assay *S. equi* bacteria donated by Dr. Wondwossen Gebreyes (collected at the OSU Veterinary Medical Center) were plated onto LB agar from a -80°C freezer and incubated at 37°C for 20 hrs. A single colony was used to inoculate 4 ml of Trypticase Soy Broth (TSB) and shaken at 37°C for 3 hours until it reached medium opacity. 1 mL of this solution was transferred to a 1.5 mL Eppendorf tube and centrifuged at 4000 g for 5 min. the solution was resuspended in 1 mL of TSB and diluted 1:4 in fresh TSB and an OD<sub>600</sub> reading was taken.

Immediately before the start of the assay, the culture was diluted such that the OD<sub>600</sub> value was 0.001 and the culture volume was at least 11 mL. 10 µL of the protein solution to be tested were added to a 96-well plate, with 3 wells for each protein concentration, and 90 µL of the bacterial suspension was added to each well. A 100 µL sample of the bacterial suspension was used as a control. The plate was then covered and shaken at 37°C overnight. After 18 hrs, the OD<sub>620</sub> reading of the wells in the plate was taken and inhibition curves were generated.

### **2.3.2 Radial Diffusion Assay**

The Radial Diffusion Bacteria were grown overnight in 20 ml of TSB for 18 hrs. 50 µL of this suspension was added to 50 mL of fresh TSB and shaken for 2.5 hrs at 37°C to reach midlogarithmic phase. A 4 ml sample of this culture was centrifuged at 900 g for 10 min at 4°C, washed once with cold 10 mM pH 7.4 sodium phosphate buffer (NAPB), and suspended in 10 mL of cold NAPB. The OD<sub>620</sub> was measured and a sample containing 4\*10<sup>6</sup> CFU was added to 10 mL of autoclaved, warm 10 mM NAPB containing 3 mg of powdered TSB media, 1% w/v low-electroendosmosis type agarose (SeaKem) and a final concentration of .02% v/v Tween 20 (Sigma). The agar mixture was vortexed and poured into a 100x15 mm Petri dish (Fischer Scientific) to a depth of ~1 mm. A template grid and a 3-mm biopsy punch were used to make 16 evenly spaced wells in the agarose layer. 5 µL of each test solution were

added to each well and the plate were incubated at 37°C for 3 hrs. At the conclusion of the incubation period, the gel was overlaid with 10 mL of warm NAPB containing a double-strength amount of TSB (6% w/v) and 1% w/v agarose. After an incubation period of 18 hrs at 37°C, the zone of inhibition around each of the wells was examined to determine the zone of clearance.

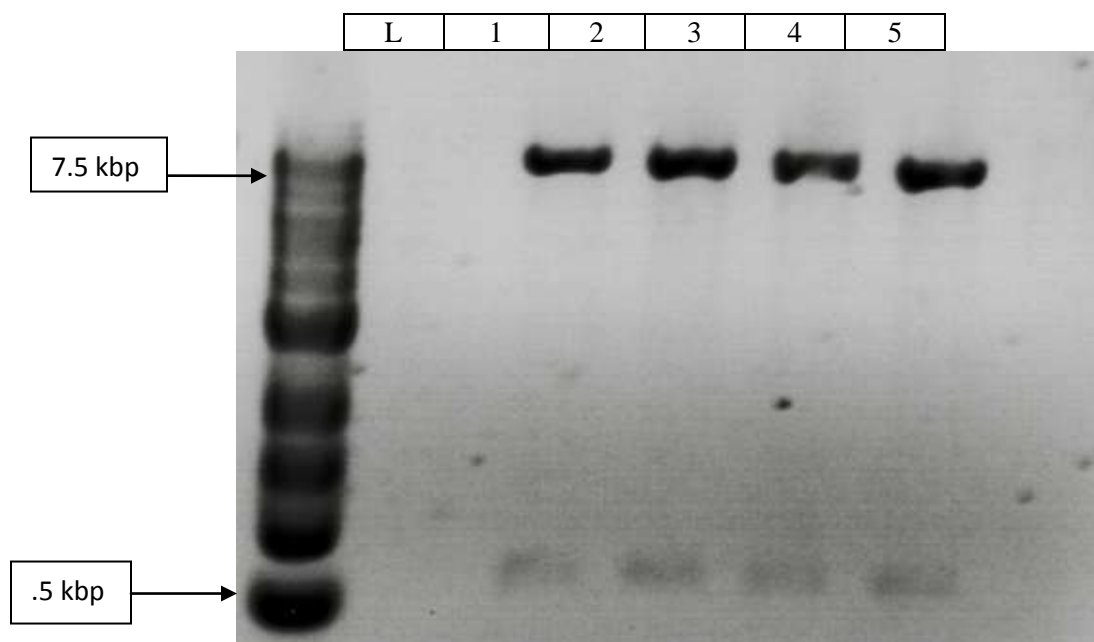
To aid in visual identification of the clearing zones, the gels were stained for 24 hrs in dilute Coomassie brilliant blue (2 mg dye, 27 mL methanol, 63 mL water and 15 ml 37% formaldehyde). The stain was decanted from the Petri dish and replaced with an aqueous solution containing 10% acetic acid and 2% DMSO and shaken for 10 min. The solution was poured off and the plates were stored for future examination. In this experiment, solutions of DEFA1, Ctr, water, a 0.1 mg/ml lysozyme control and a buffer solution control (0.4M ammonium sulfate in ½ strength cleaving buffer) were tested for activity against *E. coli*.



### 3. Results

#### 3.1 Creation of EI-Ctr

Creation of expression vectors and their introduction to *E. coli* BLR was necessary to test the method of ELP purification. After transformation of the E<sub>11</sub>I-Ctr into *E. coli* DH5 $\alpha$ , the recovered plasmids were digested with AgeI and HindIII in one digestion, and EcoRI and HindII in another. The digested fragments were separated on a 1% w/v agarose gel, seen on Figure 4. In the pET-EI-Ctr vector, the AgeI site is 40 bp behind the EcoRI site. When the plasmid is cut, the fragments excised should be roughly 526 and 7200 bp in length. For the five clones shown, which were digested with AgeI and HindII, only one was classified as “noise,” where growth was observed on an Amp<sup>+</sup> plate of bacteria without the vector containing the Amp resistance gene.



**Figure 4:** Digest of plasmids from clones possibly containing the E<sub>11</sub>I-Ctr gene. Clones 2-4 possess the pET-E<sub>11</sub>I-Ctr gene. L is a 1 Kb ladder, and lanes 1-5 contain the pET-EI-Ctr plasmids recovered in the miniprep and digested with AgeI and HindIII.

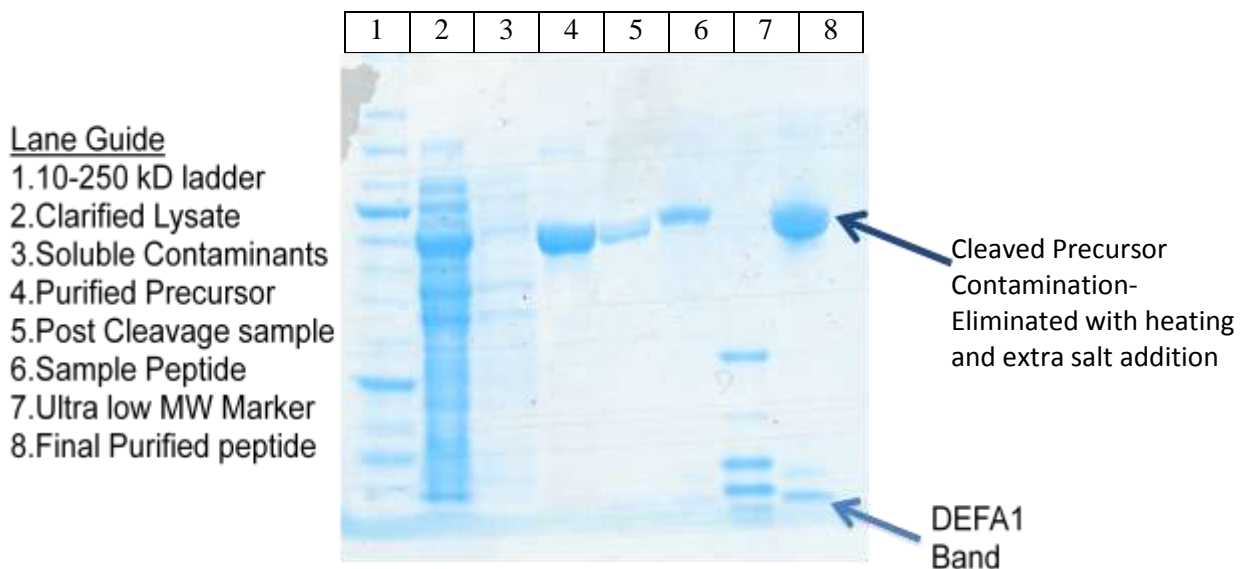
As seen in Figure 4, there were four successful clones to choose from, and the vector cut in lane 2 was used to transform the vector in *E. coli* BLR. This transformation was confirmed with a miniprep and the

sequence was verified by the DNA sequencing provided by the OSU Department of Physics. Along with DEFA1, the vector in *E. coli* BLR it can be used to produce the E<sub>11</sub>I-Ctr fusion protein.

### 3.2 Peptide Purification

Following the creation of the vectors, attempts were made to purify the peptides. After purification all of the samples taken were loaded onto Tricene-SDS 10-25% precast gels with a 10-250 kD protein ladder and an Ultra Low Molecular Weight Marker (ULMWM, Sigma) with a smallest marker size of 1 kDa. DEFA1 is approximately 4 kDa and Ctriporin 2.1 kDa. Protein concentrations were checked with a A<sub>280</sub> reading.

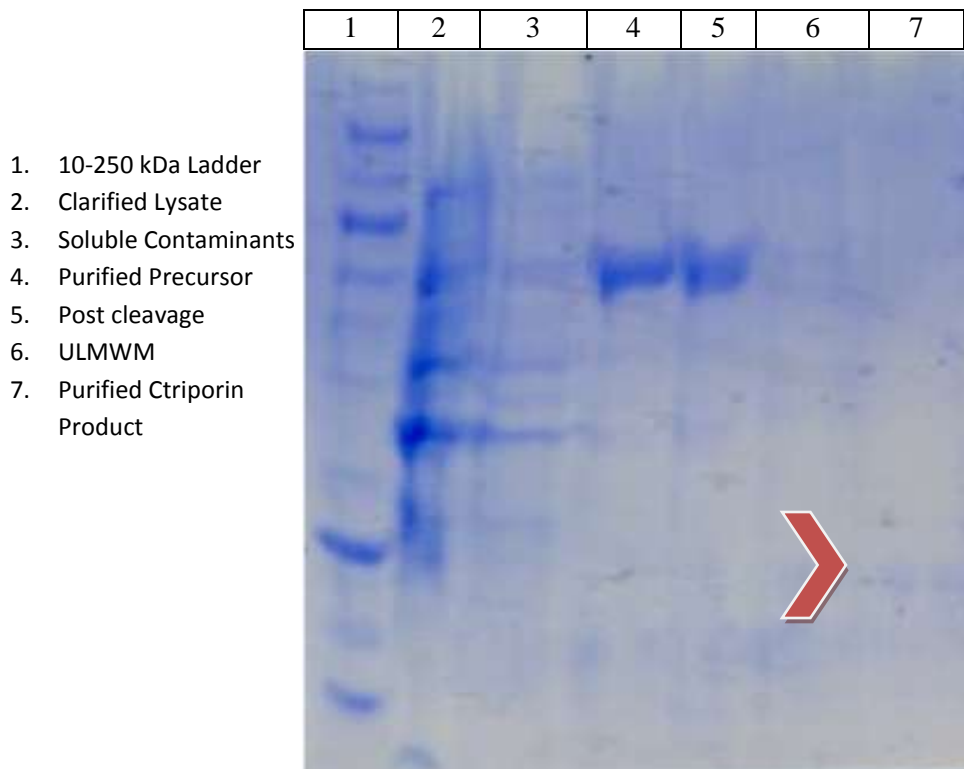
Purifying the peptides was especially difficult. With such a small size protein, it was difficult to separate the cleaved fusion protein from the sample. As seen in Figure 5 below, some of the cleaved fusion precursor still appears in the final purified peptide band. With extra salt addition, this band



**Figure 5:** Results of inverse transition cycling purification for DEFA1

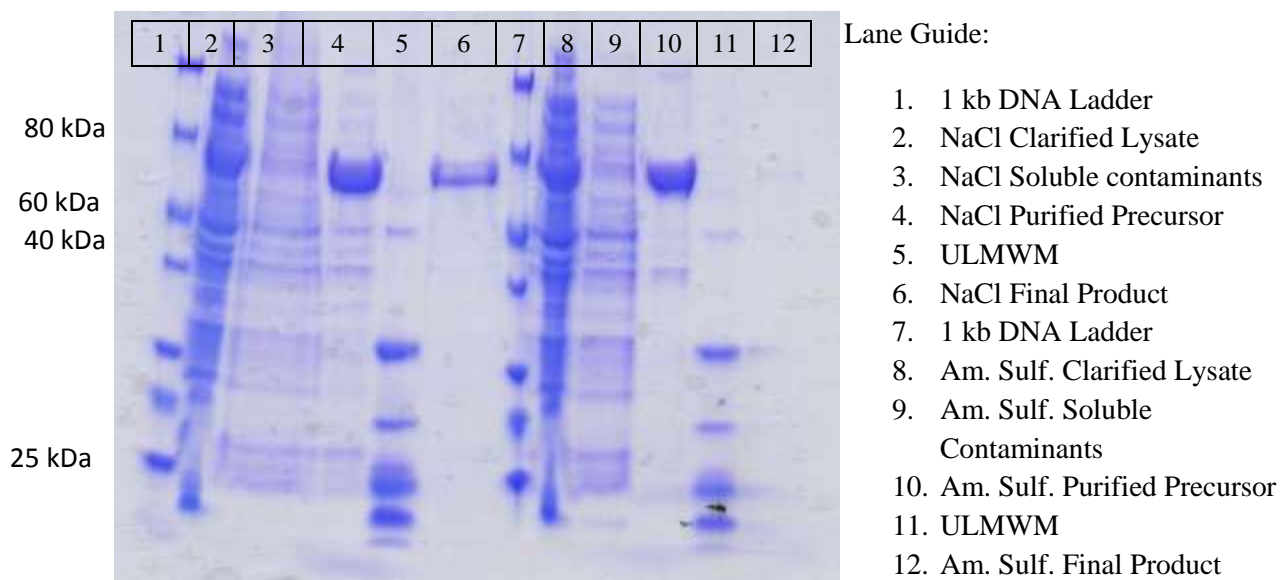
can be eliminated, but dilutes the concentration of the peptide even further. Lyophilization could increase the peptide concentration, but was unavailable for most of this research. Further, it was observed that the final peptide concentrations were sometimes too low to be observed on a Tricene-SDS gel. These results

were observed for Ctriporin purification, which may also be too small to be observed on the Tricene-SDS gel, seen in Figure 6 below. The precursor is clearly visible in Lanes 4 and 5, however only a faint band could be observed for Ctriporin. In addition, it was observed that the peptides could diffuse out of the gel during the destaining process. After the initial destain, peptide bands could be noted but the gel was left to further destain over a 24 hr period, and within an hour of the initial destain, the peptide bands had disappeared.



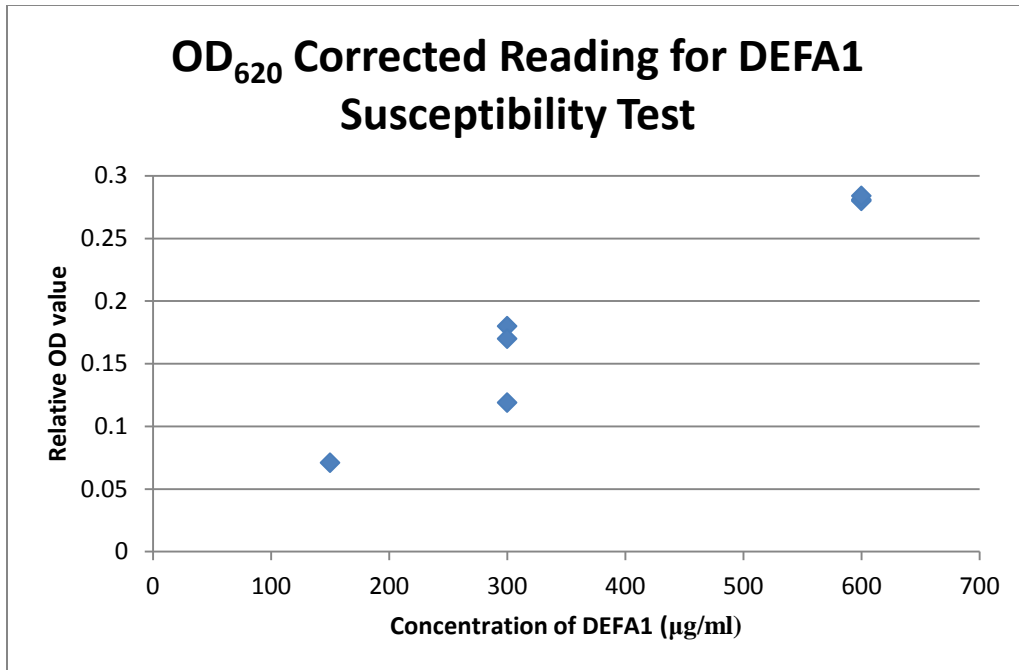
**Figure 6:** Tricine-SDS gel for purified Ctriporin. Precursor band are clearly visible in lanes 4 and 5, but the product band is incredibly faint

However, there is clearly an improvement over the original protocol using sodium chloride, as seen in Figure 7 below.



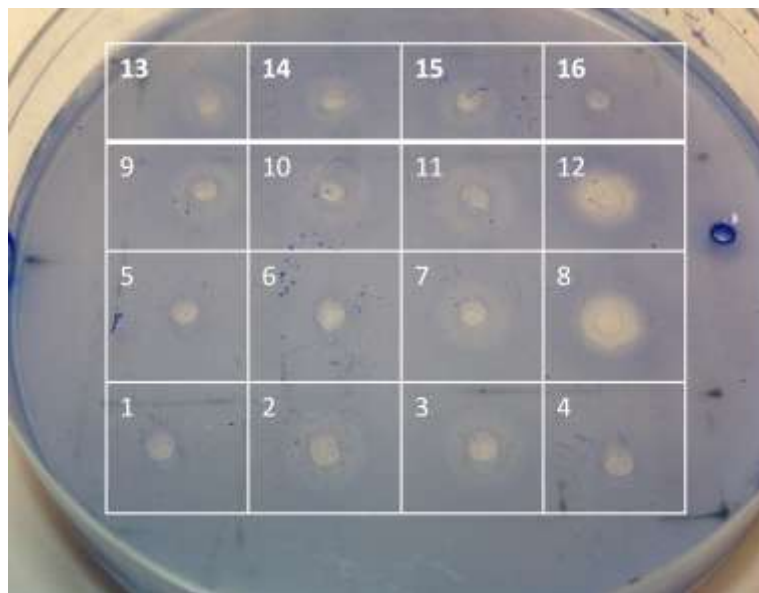
**Figure 7:** Tricine-SDS gel comparing side by side purifications of DEFA1 where NaCl is the primary salt and ammonium sulfate is the primary salt

After purification of DEFA1 to a recovered concentration of 3 mg/ml, the broth microdilution activity test was used to measure the activity of the peptide. Diluted to concentrations of 150, 300 and 600 µg/ml, values which according to the relevant literature should be lethal to the *S. equii* bacteria in the media, the results of the broth microdilution were not what was expected. Instead, the opposite effect was observed, shown in Figure 8. The OD<sub>620</sub> reading actually increased linearly with increasing concentration. The only difference in the samples was the concentration of the buffer. From literature, it is known that the components of the buffer solution for these peptides, 0.4M ammonium sulfate in ½ strength cleaving buffer, can improve bacterial growth at dilute concentrations.<sup>19</sup> This result may indicate that this buffer solution is improving bacterial growth conditions over the lethal activity of the peptide samples.



**Figure 8:** OD<sub>620</sub> readings of the DEFA1 broth microdilution susceptibility test against *S. equii*. The trend is in fact the opposite of that expected, as the lowest concentration of DEFA1 should have the highest OD reading.

The second activity test, the radial diffusion assay, is known to be more sensitive than the broth microdilution assay was used to determine activity in lieu of poor broth microdilution results. The results from the radial diffusion assay can be seen in Figure 9.



Well Assignments:

1. Blank
2. Ctriporin 6mg/ml
3. Ctriporin 6mg/ml
4. Blank
5. Water
6. Water
7. Ctriporin 6mg/ml
8. Lysozyme .1 mg/ml
9. Buffer solution
10. Buffer solution
11. Buffer solution
12. Lysozyme 0.1 mg/ml
13. DEFA1 3 mg/ml
14. DEFA1 3mg/ml
15. DEFA1 3 mg/ml
16. Water

**Figure 9:** Radial diffusion assay for both DEFA1 and Ctriporin. Well assignments can be seen to the right of the figure.

The assay works as it should, with zones of clearing visible around the lysozyme that are larger than the biopsy punch holes, indicating diffusion of the peptide into the surrounding media and lytic activity against the bacterial cells present in the media. However, the results are the similar to those of the broth microdilution assay. At first it may appear that the peptides are active as there is a radius of lighter color around the wells containing the peptide solution, if not a defined zone of clearing as with lysozyme. On closer inspection, the buffer solution (0.4M ammonium sulfate in  $\frac{1}{2}$  strength cleaving buffer) has the same approximate activity. Therefore, it cannot be said that the peptide has activity even if there is a noticeable difference between the samples with peptide and plain water. The buffer solution in for the current ELP protocol seems to have an influencing affect on the activity assays, acting both as a protagonist and antagonist on bacterial growth depending on the assay.

## 4. Conclusions

Purifying antimicrobial peptides with an ELP-intein fusion protein faces some serious challenges. First and foremost is the inability to produce an active peptide product. Until an active peptide product can be recovered the effect of a shortened tag length cannot be investigated. In both of the activity tests, the buffer solution had a major effect on the outcome of the activity assay. In one case it promoted bacterial growth with increasing concentration of the buffer. In a different assay, it caused the appearance of a false-positive test. In future experiments, the buffer must be removed or changed to remove the appearance of this false positive. Buffer exchange using an ion-exchange resin is one option currently being investigated but without results at this time. A second option is to return to the original purification protocol described in Nature, where the salt used as the precipitation agent was NaCl.<sup>12</sup> Even though the results of the purification may not be as pure, active peptide can be recovered and the impurity of cleaved precursor can be removed by filtering the sample.<sup>13</sup> In addition, *S. aureus* is known to have a high tolerance to sodium chloride, with an ability to grow normally in concentrations as high as 7.5%.<sup>20</sup> The salt concentration in the final product of the purification using NaCl is 8.7%, so the effect of the buffer on *S. aureus* may not be as prevalent as it was on *E. coli*.

A second issue with the purification may be the buffer inactivating the peptides. Ammonium sulfate may be interacting with the peptides and causing an inactivation by unknown chemical reaction or by interfering with mechanism of action of the peptides against bacterial cells. Antimicrobial peptides are recent newcomers into the field of potential antimicrobial agents and are not well understood at this point in time. The role of ions in the mechanism of action for antimicrobial peptides has not been described, and could be a reason that the ELP purification of antimicrobial peptides has been unsuccessful here. In addition, the conditions of the purification, such as temperature, could be another factor causing inactivation of the peptides, and may be play a role in the way the buffer inactivates the peptides. There are several temperatures shifts and at least one freeze thaw cycle in the ELP purification protocol, if the peptides are used immediately after purification. These could cause reaction of the peptides with the

contents of the buffer solution, which while pure, most likely contains a range of impurities and cellular components undetectable on a Tricene-SDS gel.

In summary, the purification of antimicrobial peptides using an ELP-intein fusion protein still has many unsolved problems that must be addressed even before the effect of shortening the tag length of the peptides can be examined. Buffer effects must be better understood and a secondary method of final purification, such as filtering out the cleaved precursor instead of recovering the final pure product by a salt addition, may have to be considered. With some changes, the use of an ELP-intein fusion to purify antimicrobial peptides may still be a viable method of purification.



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